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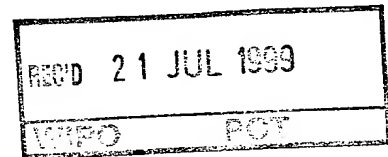
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ISOLATION, CLONING AND SEQUENCE ANALYSIS OF THE INTEGRIN
SUBUNIT $\alpha 10$, A $\beta 1$ -ASSOCIATED COLLAGEN-BINDING INTEGRIN
EXPRESSED ON CHONDROCYTES

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Abstract

We have found that chondrocytes express a novel collagen type II-binding integrin, a new member of the $\beta 1$ -integrin family. The integrin α -subunit, which has a Mr of 160 kD (reducing conditions), was isolated from bovine chondrocytes by affinity purification. The human homologue was obtained by screening a human chondrocyte library with a bovine cDNA probe. Cloning and cDNA sequence analysis of the human integrin α -subunit, designated $\alpha 10$, show that it shares the general structure of other integrin α -subunits. The predicted amino acid sequence consists of a 1167 amino acid mature protein, including a signal peptide (22 aa), a long extracellular domain (1098 aa), a transmembrane domain (25 aa) and a short cytoplasmic domain (22 aa). The extracellular part contains a seven-fold repeated sequence, an I-domain (199 aa) and three putative divalent cation-binding sites. The deduced amino acid sequence of $\alpha 10$ is 35% identical to the integrin subunit $\alpha 2$ and 37% identical to the integrin subunit $\alpha 1$. In northern blot analysis a single mRNA of 5.4 kb was detected in chondrocytes. A peptide antibody against the predicted sequence of the cytoplasmic domain of $\alpha 10$, immunoprecipitated two proteins with M_s of 125 kD and 160 kD from chondrocyte lysates under reducing conditions. The peptide antibody specifically stained chondrocytes in tissue sections of human articular cartilage showing that $\alpha 10\beta 1$ indeed is expressed in cartilage tissue.

Introduction

The integrins are a large family of transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions (1-5). All known members of this superfamily are non-covalently associated heterodimers composed of an α - and a β - subunit. At present, 8 β - ($\beta 1$ - $\beta 8$) (for references see (6)) and 16 α - subunits ($\alpha 1$ - $\alpha 9$, αv , αM , αL , αX , αIIb , αE and αD) have been characterised (6-21) and these subunits associate to generate more than 20 different integrins. The $\beta 1$ -subunit has been shown to associate with ten different α -subunits, $\alpha 1$ - $\alpha 9$ and αv , and to mediate interactions with extracellular matrix proteins such as collagens, laminins and fibronectin. The major collagen binding integrins are $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (22-25). The integrins $\alpha 3\beta 1$ and $\alpha 9\beta 1$ have also been reported to interact with collagen (26;27) although this interaction is not well understood (28). The extracellular N-terminal regions of the α and β integrin subunits are important in the binding of ligands (29;30). The N-terminal region of the α -subunits is composed of a seven-fold repeated sequence (12;31) containing FG and GAP consensus sequences. The repeats are predicted to fold into a β -propeller domain (32) with the last three or four repeats containing putative divalent cation binding sites. The α -integrin subunits $\alpha 1$, $\alpha 2$, αD , αE , αL , αM and αX contain a ~200 amino acid inserted domain, the I-domain (A-domain), which shows similarity to sequences in von Willebrand factor, cartilage matrix protein and complement factors C2 and B (33;34). The I-domain is localised between the second and third FG-GAP repeats, it contains a metal ion-dependent adhesion site (MIDAS) and it is involved in binding of ligands (35-38)

Chondrocytes, the only type of cells in cartilage, express a number of different integrins including $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ (39-41). We have shown that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ mediate chondrocyte interactions with collagen type II (25) which is one of the major components in cartilage. We have also shown that $\alpha 2\beta 1$ is a receptor for the

cartilage matrix protein chondroadherin (42). In the present study we have isolated a novel collagen type II binding integrin, $\alpha 10\beta 1$, from bovine articular chondrocytes. Cloning and sequence analysis of the human homologue is described and expression of $\alpha 10$ on chondrocytes is examined.

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Materials and Methods

Antibodies

A polyclonal antiserum was generated against the $\alpha 10$ cytoplasmic domain peptide CKKIPEEEKREEKLE. Peptide synthesis, conjugation to KLH, injection of rabbits and affinity purification were performed by Innovagen AB (Lund, Sweden). Monoclonal antibodies against human integrin subunit $\beta 1$ (P4C10), $\alpha 2$ (P1E6) and $\alpha 3$ (P1B5) (unpurified ascites fluid) were from Life Technology Inc, Grand Island NY, USA. The monoclonal antibody against human integrin subunit $\alpha 1$ (TS2/7; hybridoma supernatant) was a kind gift from Timothy Springer, Boston Blood Center, Boston, MA (43). Polyclonal antiserum against the rat $\beta 1$ -integrin subunit was kindly provided by Staffan Johansson, Uppsala, Sweden (44). Polyclonal antibodies (serum) against human integrin subunits $\alpha 2$ (AB1936), $\alpha 3$ (AB1920) and polyclonal antibody (serum) against rat integrin subunits $\alpha 1$ (AB1934) were from Chemicon International Inc. Polyclonal antibodies against the integrin subunit $\alpha 9$ (affinity purified IgG) were a kind gift from Dean Sheppard, UCSF- Lung Biology Center, San Francisco, CA (6).

Cell isolation and culture

Bovine chondrocytes were isolated by digestion of articular cartilage from 4- 6 month old calves with collagenase (CLS1, Worthington) as described elsewhere (45). Briefly, cartilage slices were digested by collagenase in EBSS (Earle's balanced salt solution, GIBCO) for 15-16 hours at 37 °C. The tissue digest was filtered through a 100 μ m nylon filter and the isolated cells were then washed three times in Dulbecco's modified phosphate buffered saline (PBS, GIBCO). Human chondrocytes from articular cartilage were isolated by digestion with pronase (Calbiochem) for 1 hr followed by collagenase (Boehringer Mannheim) for 15-18 hrs, as

described by Häuselmann *et al* (46). The cells were filtered and washed as described above.

Human chondrocytes were cultured in Dulbeccos MEM and F12 (1:1) supplemented with 10%

fetal calf serum, 25 µg/ml ascorbic acid, 50 UI penicillin and 50 µg/ml streptomycin (GIBCO).

To harvest cells, the culture dish was washed three times with Ca/Mg-free PBS and the cells were incubated with 0.5% trypsin and 1 mM EDTA (GIBCO) in PBS (-Ca,Mg) for 5 minutes

Detached cells were suspended in medium containing 10 % FCS or in PBS containing 1mg/ml trypsin inhibitor (Sigma) and then washed in PBS.

Coupling of affinity columns

Collagen type II isolated from nasal cartilage by pepsin digestion (47) was coupled to CNBr-Sepharose (Pharmacia Biotech, Uppsala, Sweden) according to the published procedure (25).

A control column was produced by treating CNBr-Sepharose in a similar manner but in the absence of protein. Bovine fibronectin (Sigma) was coupled to CNBr-Sepharose according to instructions from the manufacturer. After blocking, the fibronectin-Sepharose was washed three times with PBS.

Affinity purification and immunoprecipitation of ¹²⁵I-labeled membrane proteins

Human chondrocyte cell surface proteins were ¹²⁵I-labeled and affinity purified on collagen type II-Sepharose according to the published procedure (25). Cell-lysates or affinity purified samples were immunoprecipitated as described earlier (42). The following antibodies were used in immunoprecipitation experiments: monoclonal antibodies against the human integrin subunits β1, α1, α2 or α3 (unpurified ascites fluid, dilution 1/100); polyclonal antibody against the rat integrin subunit β1 (purified IgG, 50-100 µg/ml); polyclonal peptide antibodies against the integrin subunits α1, α2, α3 and α10 (antiserum, dilution 1/100). The immunoprecipitated

proteins were separated by 4-12% SDS-PAGE and visualized by image analysis using the BioImaging Analyzer Bas2000 (Fuji Photo Film Co., Tokyo Japan).

Affinity purification of the integrin subunit $\alpha 10$ on collagen type II-Sepharose

Freshly isolated bovine chondrocytes (2500×10^6) were lysed in 6 ml of 1 % Triton X-100, 10 mM Tris-HCl, pH 7.4, 100 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 1 mM PMSF, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ for 1 hour on ice. The lysate was centrifuged for 30 minutes at 10,000 rpm and the pellet was discarded. Collagen type II-Sepharose (4 ml) and the fibronectin-Sepharose (2 ml) were equilibrated with at least 20 volumes of 0.1 % Triton X-100, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ (equilibration buffer). The cell-lysate was passed over the fibronectin-Sepharose twice and then incubated with the collagen-Sepharose end over end for 3 hours. The columns were washed (15 gel volumes) with the equilibration buffer containing 75 mM NaCl and bound proteins were eluted (10 x 0.5 ml) with 20 mM EDTA, 1 mM PMSF and 10 mM Tris-HCl, pH 7.4.

Isolation of internal peptides by in-gel digestion and peptide sequencing

Affinity purified proteins were concentrated by precipitation using the methanol/chloroform protocol (48). After reduction/alkylation with DTT/Iodoacetamide (49), samples were subjected to SDS-PAGE on a 4-12 % polyacrylamide gel and protein bands were visualized by Coomassie staining. The 160 kD protein-band was excised from the gel and prepared for in-gel digestion (50). Briefly, the gel slice was washed extensively to remove SDS and the dye, and after complete drying, protease was forced into the gel by rehydration with a solution of either Endoproteinase Lys C (Wako, Neuss, Germany) or modified trypsin (Promega Madison,

WI) in the appropriate digestion buffer. Following an overnight incubation, peptides were extracted and then isolated by narrow bore reversed-phase liquid chromatography on a μ RPC C2/C18 SC 2.1/10 column, operated in a SMART System (Pharmacia Biotech, Uppsala, Sweden). Several peptides were analyzed by Edman degradation in a Perkin-Elmer Applied Biosystem Model 476 sequencer, operated according to the manufacturer's instructions.

mRNA purification and cDNA-synthesis

mRNA from bovine or human chondrocytes were isolated using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized at 42° C for 1hr using the Superscript™ II RNase H Reverse Transcriptase cDNA Synthesis system (GIBCO-BRL), random DNA hexamers and oligo (dT) (Promega, Madison, WI).

PCR amplification

PCR-reactions were performed in 50 μ l reaction volumes and contained 1 x Taq polymerase buffer (GIBCO-BRL), 1.5 mM MgCl₂, 1 μ M of each primer, 0.025 U/ μ l Taq polymerase, 1 μ l of DNA template (bovine chondrocyte cDNA) and 0.1 mM each of dATP, dGTP, dCTP and dTTP (Boehringer Mannheim). PCR-samples were heated to 94°C for 5 minutes in a thermocycler and then subjected to 35 cycles consisting of 30 seconds at 94°C (denaturation), 30 seconds at 48°C or 52°C (annealing) and 3 minutes at 72°C (extension). The PCR-products were re-amplified using 1 μ l of each product for an additional 35 cycles. Amplified DNA was analyzed by 1 % agarose gel electrophoresis. Small DNA fragments were analyzed using 4% metha phore agarose (FMC BioProducts).

The degenerate primers GAY AAY ACI GCI CAR AC (DNTAQT, forward) and TIA TIS

WRT GRT GIG GYT (EPHHSI, reverse) were used in PCR to amplify the nucleotide sequence corresponding to the bovine peptide 1 (Table I). A 900 bp-PCR-fragment was then amplified from bovine cDNA using an internal specific primer TCA GCC TAC ATT CAG TAT (SAYIQY, forward) corresponding to the cloned nucleotide sequence of peptide 1 together with the degenerate primer ICK RTC CCA RTG ICC IGG (PGHWDR, reverse) corresponding to the bovine peptide 2 (Table I). Mixed bases were used in positions that were twofold degenerate and inosines were used in positions that are three- or fourfold degenerate.

To obtain cDNA that encoded the 5' end of $\alpha 10$ we designed the primer AAC TCG TCT TCC AGT GCC ATT CGT GGG (reverse; residue 1254-1280 in $\alpha 10$ cDNA) and used it for rapid amplification of the cDNA 5' end (RACE) as described in the Marathon[™] cDNA

Amplification kit (Clontech Intermedica).

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Cloning and sequencing of cDNA

PCR-fragments were isolated and purified from agarose gels using Jet Sorb DNA extraction kit (Saveen). Purified fragments were then cloned with the Pcr Script[™] Sk(+) kit (Stratagene). Selected plasmides were purified from liquid cultures using QIAGEN-plasmid midi preparation kit (QIAGEN Inc.) and sequenced by ABI 373A- sequencer using ABI Prism[™] Dye Terminator Cycle Sequencing Core kit (PERKIN ELMER) together with T3, T7 and internal specific primers.

Library screening

The cloned 900bp PCR-fragment, corresponding to bovine $\alpha 10$ -integrin, was digoxigenin-labeled according to the DIG DNA labeling kit (Boehringer Mannheim) and used as a probe

for screening of a human articular chondrocyte λ ZapII cDNA library (provided by Dr. Michael Bayliss) (51). Positive clones containing the pBluescript SK⁺ plasmid with the cDNA insert

were rescued from the ZAP vector by *in vivo* excision as described in the ZAP-cDNA® synthesis kit (Stratagene). Selected plasmides were purified and sequenced as described earlier using T3, T7 and internal specific primers.

Northern blot analysis

Bovine chondrocyte mRNA was purified using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden), separated on a 1 % agarose-formaldehyde gel, transferred to nylon membranes and immobilized by UV crosslinking. cDNA-probes were ³²P-labelled with Random Primed DNA Labeling Kit (Boehringer Mannheim). Filters were prehybridized for 2-4 hours at 42 °C in 5x SSE, 5x Denharts solution, 0.1 % SDS, 50 µg/ml salmon sperm DNA and 50% formamide and then hybridized over night at 42 °C with the same solution containing the specific probe (0.5-1 x 10⁶ cpm/ml). Specifically bound cDNA-probes were analyzed using a phosphoimage system (Fuji). Filters were stripped by washing in 0.1% SDS, for 1h at 80°C prior to re-probing. The α 10-integrin cDNA-probe was isolated from the rac1-containing plasmid using the restriction enzymes BamHI (GIBCO-BRL) and NcoI (Boehringer Mannheim). The rat β 1-integrin cDNA probe was a kind gift from Staffan Johansson, Uppsala, Sweden (25).

Tissue staining

Human cartilage from the trochlear groove, obtained during surgery, was provided by Dr. Anders Lindahl, Sahlgrenska University Hospital, Gothenburg, Sweden. Frozen sections of cartilage tissue were fixed in acetone at -18°C for 5 minutes, washed in PBS and then treated

with 2mg/ml of hyaluronidase (Sigma) in PBS, pH 5.0 for 15 minutes at 37°C. After washing with PBS, sections were blocked for 15 minutes at room temperature in 1% H₂O₂ in PBS to remove endogenous peroxidase activity. Sections were then washed in PBS, blocked with 0.5% casein and 0.05% thimerosal in PBS (blocking buffer) for 15 minutes at room temperature and then incubated over night at 4°C with the affinity purified antibodies against the integrin subunits $\alpha 9$ and $\alpha 10$ (5 μ g/ml in blocking buffer). For control, $\alpha 10$ -antibody was preincubated with the $\alpha 10$ peptide (0.1mg/ml) for 30 minutes at 4°C. After washing in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc; diluted 1:200 in blocking buffer) at room temperature for 60 minutes. Washed sections were then incubated with vectastain ABC reagent (Vector Laboratories, Inc.) for 1 h at room temperature, washed, and the color was developed using 1 mg/ml of DAB in 0.1M Tris-HCl, pH 7.2 and 0.02 % H₂O₂. Sections were rinsed in water for 5 minutes followed by 75 % ethanol, 95 % ethanol and 99.5 % ethanol for 5 minutes each, and then three times in xylene for 3 minutes at room temperature. Samples were mounted in Pertex (HISTOLAB Products AB, Gothenburg, Sweden).

Results

Identification and isolation of the chondrocyte $\alpha 10$ integrin subunit

Affinity purification of ^{125}I -labelled membrane proteins from human chondrocytes on collagen type II-Sepharose followed by immunoprecipitation showed that these cells, in addition to $\alpha 1\beta 1$ and $\alpha 2\beta 1$, express an unidentified $\beta 1$ -related α subunit (Figure 1). This integrin subunit had a molecular weight of approximately 160 kD and was slightly larger than the $\alpha 2$ integrin subunit. This finding is in agreement with a previous study showing that bovine chondrocytes also express an unidentified collagen binding $\beta 1$ -associated α -subunit of similar M_r (25). To isolate this protein, we affinity purified collagen type II-binding proteins from bovine chondrocyte since. The cell lysate was applied to a fibronectin-Sepharose precolumn followed by a collagen type II-Sepharose column. As shown in Figure 2, a number of proteins were eluted from the affinity columns, however, a protein with M_r of approximately 160 kD was specifically eluted with EDTA from the collagen column but not from the fibronectin column. The M_r of this protein corresponded with the M_r of the unidentified $\beta 1$ -related integrin subunit (Figure 1). The 160 kD protein band was excised from the SDS-PAGE gel, digested with trypsin or Endoproteinase Lys C and several of the isolated peptides were analyzed. Table 1 shows the amino acid sequence of 6 individual peptides.

Cloning and sequencing of the human integrin α -subunit homologue

The nucleotide sequence of peptide 1 (Table 1) was obtained by PCR-amplification, cloning and sequencing of bovine cDNA. From this nucleotide sequence an exact primer was designed and then applied in PCR-amplification with degenerate primers corresponding to peptides 2-6 (Table I). Primers corresponding to peptides 1 and 2 amplified a 900 bp PCR-fragment from bovine cDNA which was cloned, sequenced and then used for screening of a human articular

chondrocyte λ ZapII cDNA library to obtain the human integrin α -subunit homologue. Two overlapping clones, hc1 and hc2 (Figure 3), were isolated, subcloned and sequenced. These clones contained 2/3 of the nucleotide sequence including the 3' end of the cDNA. A third clone (rac1; Figure 3), containing the 5' end of the α 10 cDNA, was obtained using the RACE technique. From these three overlapping clones of α 10 cDNA, 3884 nucleotides were sequenced (Figure 4). The sequence contains a 3504-nucleotide open reading frame that is predicted to encode a 1167 amino acid mature protein. The predicted sequence included a signal peptide (22 aa), a long extracellular domain (1098 aa), a transmembrane domain (25 aa) and a short cytoplasmic domain (22 aa). Sequence analysis of the 160 kD protein sequence showed that it was a member of the integrin α -subunit family and the protein was named α 10.

Comparison of α 10 integrin subunit with other α -subunits

Alignment of α 10 with known α subunits showed that its structure follows the conserved pattern of integrin α subunits (Figure 5). The extracellular domain contains a seven-fold repeated sequence including FG and GAP consensus sequences, three putative divalent cation-binding sites (DxD/NxD/NxxxD) and an I-domain of 199 amino acids. The protein contains 10 potential N-linked glycosylation sites (NXT/S). The calculated molecular weight is 153 kD if carbohydrate chains with an average molecular weight of 2.5 kD are assumed to attach to all 10 putative glycosylation sites. This is in agreement with the molecular weight of α 10 as judged by SDS-PAGE where the M_r was estimated to 160 kD. In contrast to most α -integrin-subunits the cytoplasmic domain does not contain the conserved sequence KxGFF (R/K) R (Table II). Instead the predicted amino acid sequence is KLGFFAH. The deduced amino acid sequence of α 10 showed the highest identity to the

collagen binding integrin subunits $\alpha 1$ (37%) and $\alpha 2$ (35%). The similarity of integrin α subunits are shown in Figure 6.

Expression of the $\alpha 10$ integrin subunit on chondrocytes

Northern blot analysis of mRNA from bovine chondrocytes showed that a human $\alpha 10$ cDNA-probe hybridized with a single mRNA of approximately 5.4 kb (Figure 7). As a comparison, a cDNA-probe corresponding to the integrin subunit $\beta 1$ was used. This cDNA-probe hybridized a mRNA-band of approximately 3.5 kb on the same filter. Translation of the $\alpha 10$ nucleotide sequence revealed an open reading frame of 3504 nucleotides (Figure 4) which indicates that around 2000 nucleotides in the mRNA is not translated.

To study expression of $\alpha 10$ on protein level, ^{125}I labelled membrane proteins from human chondrocytes were immunoprecipitated with polyclonal antibodies against the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 10$ (Figure 8). A polyclonal peptide antibody, raised against the cytoplasmic domain of $\alpha 10$, precipitated two protein bands with M_r of 160 kD and 125 kD under reducing conditions. The $\alpha 10$ associated β -chain migrated as the $\beta 1$ integrin subunit both under reducing and non-reducing conditions (Figures 8a,b).

Expression of $\alpha 10$ in cartilage was examined by immunostaining of human articular cartilage from the trochlear groove with the polyclonal $\alpha 10$ -antibody. As shown in Figure 9, this antibody specifically stained the chondrocytes in the cartilage tissue sections. The staining was completely abolished when the antibody was preincubated with the $\alpha 10$ -peptide. A control antibody against the $\alpha 9$ integrin subunit did not stain chondrocytes in the tissue sections (Figure 9).

Discussion

We show in this study that human chondrocytes express a novel, $\beta 1$ -associated, collagen type II-binding integrin, in addition to integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. We have, in an earlier study, presented some evidence for that bovine chondrocytes and human chondrosarcoma cells also express an unknown integrin (25). Bovine chondrocytes were readily available and therefore used in the isolation of the unknown integrin subunit.

In the affinity purification experiment, fibronectin-Sepharose was used as a precolumn since earlier studies showed that fibronectin is not a ligand for this integrin (data not shown). As shown in Figure 2, several proteins were eluted from the affinity columns. It was difficult to interpret the protein pattern as typical integrin bands were not clearly distinguished on the SDS-PAGE gel. Based upon the findings that the unknown chondrocyte integrin α -subunit in the immunoprecipitation experiment (Figure 1) had a M_r of 160 kD, a protein with similar M_r , which was specifically eluted with EDTA from the collagen type II-column, was excised from the gel and used for peptide sequencing. The difficulty to distinguish integrin bands on the gel may be explained by that most integrins eluted from the columns were partially degraded although a mixture of protease inhibitors were included in the lysate buffer. The novel $\alpha 10$ integrin subunit appeared resistant to proteolytic degradation. The immunoprecipitation experiments showed that $\alpha 2$ and the new α integrin subunit have similar M_r s under reducing conditions (Figure 1). To avoid contamination of $\alpha 2$, the 160 kD-protein was excised from the SDS-PAGE gel as a very narrow band. It likely contained one protein since human homologues to all six bovine peptides (Table 1) that were isolated from the 160 kD protein were found in the predicted amino acid sequence of human $\alpha 10$ subunit (Figure 4).

The deduced amino acid sequence of $\alpha 10$ shares the general structure of the integrin α -subunits described in previously published reports (6-21). The large extracellular N-terminal

part of $\alpha 10$ contains a seven-fold repeated sequence which was recently predicted to fold into a β -propeller domain (32). The integrin subunit $\alpha 10$ contains three putative divalent cation-binding sites (DxD/NxD/NxxxD) (52), a single spanning transmembrane domain and a short cytoplasmic domain. In contrast to most α -integrin subunits the cytoplasmic domain of $\alpha 10$ does not contain the conserved sequence KxGFF (R/K) R. The predicted amino acid sequence in $\alpha 10$ is KLGFFAH. Several reports indicate that the integrin cytoplasmic domains are crucial in signal transduction (53) and that membrane-proximal regions of both α - and β -integrin cytoplasmic domains are involved in modulating conformation and affinity state of integrins (54-56). It is suggested that the GFFKR motif in α -chains are important for association of integrin subunits and for transport to the plasma membrane (57). The KxGFFKR domain has been shown to interact with the intracellular protein calreticulin (58) and interestingly, calreticulin-null embryonic stem cells are deficient in integrin-mediated cell adhesion (59). It is, in this context, tempting to speculate that the sequence KLGFFAH in $\alpha 10$ may have a key function in regulating the affinity between $\alpha 10\beta 1$ and collagen.

Integrin α subunits are known to share an overall identity of 20-40% (60). Sequence analysis showed that the $\alpha 10$ subunit is most closely related to the I-domain containing α -subunits (Figure 6) with the highest identity to $\alpha 1$ (37%) and $\alpha 2$ (35%). The integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are known receptors for both collagens and laminins (24;61;62) and we have also recently demonstrated that $\alpha 2\beta 1$ interacts with the cartilage protein chondroadherin (42). Since $\alpha 10\beta 1$ was isolated on a collagen type II-Sepharose we know that collagen type II is a ligand for $\alpha 10\beta 1$. We have also shown by affinity purification experiments that $\alpha 10\beta 1$ interacts with collagen type I (data not shown) but it remains to be seen whether laminin or chondroadherin are also ligands for this integrin.

We and others have shown previously that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are present on isolated chondrocytes although $\alpha 2\beta 1$ is found in only minor amounts (25). The peptide antibody that we raised against the cytoplasmic domain of $\alpha 10$ immunoprecipitated two proteins from human chondrocytes with M_r of 125 kD and 160 kD. The M_r of 160 kD correlates with the unidentified $\beta 1$ -associated α -subunit that was affinity purified on collagen type II-Sepharose. Immunohistochemistry using the $\alpha 10$ -antibody showed staining of the chondrocytes in tissue sections of human articular cartilage. The antibody staining was clearly specific since preincubation of the antibody with the $\alpha 10$ -peptide completely abolished the staining. An antibody against the integrin subunit $\alpha 9$ was used as a control in these experiments we (6). This integrin is a receptor for tenascin-C (63) and is not known to be present in cartilage. The immunohistochemical experiments also clearly demonstrate that $\alpha 10\beta 1$ is present not only on isolated cells but also in tissue sections of human cartilage. Taken together, we have isolated and characterized a novel collagen type II-binding integrin designated $\alpha 10\beta 1$. The $\alpha 10$ subunit was isolated from bovine chondrocytes and the human homologue was cloned and sequenced. Antibodies against the $\alpha 10$ -integrin subunit stained chondrocytes in tissue sections of articular cartilage indicating that $\alpha 10\beta 1$ is one of the collagen type II binding integrins expressed in cartilage. It is very likely that this integrin is important in the communication between chondrocytes and the extracellular matrix.

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References

1. Springer, T.A. (1990) *Nature* **346**, 425-434
2. Ruoslahti, E. (1991) *J.Clin.Invest.* **87**, 1-5
3. Hynes, R.O. (1992) *Cell* **69**, 11-25
4. Hemler, M.E. (1988) *Immunol.Today* **9**, 109-113
5. Yamada, K.M. (1991) *J.Biol.Chem.* **266**, 12809-12812
6. Palmer, E.L., Ruegg, C., Ferrando, R., Pytela, R., and Sheppard, D. (1993) *J.Cell Biol.* **123**, 1289-1297
7. Takada, Y., Elices, M.J., Crouse, C., and Hemler, M.E. (1989) *EMBO J.* **8**, 1361-1368
8. Poncz, M., Eisman, R., Heidenreich, R., Silver, S.M., Vilaire, G., Surrey, S., Schwartz, E., and Bennett, J.S. (1987) *J.Biol.Chem.* **262**, 8476-8482
9. Larson, R.S., Corbi, A.L., Berman, L., and Springer, T. (1989) *J.Cell Biol.* **108**, 703-712
10. Corbi, A.L., Kishimoto, T.K., Miller, L.J., and Springer, T.A. (1988) *J.Biol.Chem.* **263**, 12403-12411
11. Argraves, W.S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M.D., and Ruoslahti, E. (1987) *J.Cell Biol.* **105**, 1183-1190
12. Corbi, A.L., Miller, L.J., O'Connor, K., Larson, R.S., and Springer, T.A. (1987) *EMBO J.* **6**, 4023-4028

13. Briesewitz, R., Epstein, M.R., and Marcantonio, E.E. (1993) *J.Biol.Chem.* **268**, 2989-2996

14. Ziober, B.L., Vu, M.P., Waleh, N., Crawford, J., Lin, C.S., and Kramer, R.H. (1993) *J.Biol.Chem.* **268**, 26773-26783
15. Hogervorst, F., Kuikman, I., van Kessel, A.G., and Sonnenberg, A. (1991) *Eur.J.Biochem.* **199**, 425-433
16. Takada, Y. and Hemler, M.E. (1989) *J.Cell Biol.* **109**, 397-407
17. Takada, Y., Murphy, E., Pil, P., Chen, C., Ginsberg, M.H., and Hemler, M.E. (1991) *J.Cell Biol.* **115**, 257-266
18. Van der Vieren, M., Le Trong, H., Wood, C.L., Moore, P.F., St.John, T., Staunton, D.E., and Gallatin, W.M. (1995) *Immunity.* **3**, 683-690
19. Schnapp, L.M., Breuss, J.M., Ramos, D.M., Sheppard, D., and Pytela, R. (1995) *J.Cell Sci.* **108**, 537-544
20. Shaw, S.K., Cepek, K.L., Murphy, E.A., Russell, G.J., Brenner, M.B., and Parker, C.M. (1994) *J.Biol.Chem.* **269**, 6016-6025
21. Suzuki, S., Argraves, W.S., Arai, H., Languino, L.R., Pierschbacher, M.D., and Ruoslahti, E. (1987) *J.Biol.Chem.* **262**, 14080-14085
22. Ignatius, M.J., Large, T.H., Houde, M., Tawil, J.W., Barton, A., Esch, F., Carbonetto, S., and Reichardt, L.F. (1990) *J.Cell Biol.* **111**, 709-720
23. Gullberg, D., Gehlsen, K.R., Turner, D.C., Åhlén, K., Zijenah, L.S., Barnes, M.J., and

Rubin, K. (1992) *EMBO J.* 11, 3865-3873

-
24. Staaaz, W.D., Rajpara, S.M., Wayner, E.A., Carter, W.G., and Santoro, S.A. (1989) *J.Cell Biol.* 108, 1917-1924
25. Holmvall, K., Camper, L., Johansson, S., Rubin, K., Kimura, J.H., and Lundgren-Åkerlund, E. (1995) *Exp.Cell Res.* 221, 496-503
26. Forsberg, E., Ek, B., Engström, Å., and Johansson, S. (1994) *Exp.Cell Res.* 213, 183-190
27. Wayner, E.A. and Carter, W.G. (1987) *J.Cell Biol.* 105, 1873-1884
28. Weitzman, J.B., Pasqualini, R., Takada, Y., and Hemler, M.E. (1993) *J.Biol.Chem.* 268, 8651-8657
29. Elices, M.J. and Hemler, M.E. (1989) *Proc.Natl.Acad.Sci.U.S.A.* 86, 9906-9910
30. Languino, L.R., Colella, S., Zanetti, A., Andrieux, A., Ryckewaert, J.J., Charon, M.H., Marchisio, P.C., Plow, E.F., Ginsberg, M.H., Marguerie, G., and et al (1989) *Blood* 73, 734-742
31. Tuckwell, D.S., Humphries, M.J., and Brass, A. (1994) *Cell Adhes.Comm.* 2, 385-402
32. Springer, T.A. (1997) *Proc.Natl.Acad.Sci.U.S.A.* 94, 65-72
33. Colombatti, A., Bonaldo, P., and Doliana, R. (1993) *Matrix* 13, 297-306
34. Lee, C.H., Bradley, G., and Ling, V. (1995) *Cell Growth Differ.* 6, 347-354
35. Calderwood, D.A., Tuckwell, D.S., and Humphries, M.J. (1995) *Biochem.Soc.Trans.*

36. Kern, A., Eble, J., Golbik, R., and Kuhn, K. (1993) *Eur.J.Biochem.* 215, 151-159
37. Tuckwell, D.S., Reid, K.B., Barnes, M.J., and Humphries, M.J. (1996) *Eur.J.Biochem.* 241, 732-739
38. Kamata, T. and Takada, Y. (1994) *J.Biol.Chem.* 269, 26006-26010
39. Dürr, J., Goodman, S., Potocnik, A., von der Mark, H., and von der Mark, K. (1993) *Exp.Cell Res.* 207, 235-244
40. Salter, D.M., Hughes, D.E., Simpson, R., and Gardner, D.L. (1992) *Br.J.Rheumatol.* 31, 231-234
41. Woods, V.L.J., Schreck, P.J., Gesink, D.S., Pacheco, H.O., Arniel, D., Akeson, W.H., and Lotz, M. (1994) *Arthritis Rheum.* 37, 537-544
42. Camper, L., Heinegård, D., and Lundgren-Åkerlund, E. (1997) *J.Cell Biol* 138, 1159-1167
43. Hemler, M.E., Sanchez Madrid, F., Flotte, T.J., Krensky, A.M., Burakoff, S.J., Bhan, A.K., Springer, T.A., and Strominger, J.L. (1984) *J.Immunol.* 132, 3011-3018
44. Bottger, B.A., Hedin, U., Johansson, S., and Thyberg, J. (1989) *Differentiation.* 41, 158-167
45. Sommarin, Y. and Heinegård, D. (1983) *Biochem.J.* 214, 777-784
46. Häuselmann, H.J., Aydelotte, M.B., Schumacher, B.L., Kuettner, K.E., Gitelis, S.H., and Thonar, E.J.M.A. (1992) *Matrix* 12, 116-129

47. Miller, E.J. (1972) *Biochemistry* 11, 4903-4909
-
48. Wessel, D. and Flugge, U.I. (1984) *Anal.Biochem.* 138, 141-143
49. Blobel, G. and Dobberstein, B. (1975) *J.Cell Biol.* 67, 835-851
50. Hellman, U. (1997) in *Protein structure analysis. Preparation, characterization, and microsequencing* (Kamp, R.M., Choli-Papadopoulou, T., and Wittmann-Liebold, B., eds) pp. 97-104, Spriner-Verlag, Heidelberg
51. Charles, I.G., Palmer, R.M., Hickery, M.S., Bayliss, M.T., Chubb, A.P., Hall, V.S., Moss, D.W., and Moncada, S. (1993) *Proc.Natl.Acad.Sci.U.S.A.* 90, 11419-11423
52. Tuckwell, D.S., Brass, A., and Humphries, M.J. (1992) *Biochem.J.* 285, 325-331
53. Dedhar, S. and Hannigan, G.E. (1996) *Curr.Opin.Cell Biol.* 8, 657-669
54. Hughes, P.E., O'Toole, T.E., Ylanne, J., Shattil, S.J., and Ginsberg, M.H. (1995) *J.Biol.Chem.* 270, 12411-12417
55. Puzon McLaughlin, W., Yednock, T.A., and Takada, Y. (1996) *J.Biol.Chem.* 271, 16580-16585
56. O'Toole, T.E., Katagiri, Y., Faull, R.J., Peter, K., Tamura, R., Quaranta, V., Loftus, J.C., Shattil, S.J., and Ginsberg, M.H. (1994) *J.Cell Biol.* 124, 1047-1059
57. De Melker, A.A., Kramer, D., Kuikman, I., and Sonnenberg, A. (1997) *Biochem J* 529-537
58. Rojiani, M.V., Finlay, B.B., Gray, V., and Dedhar, S. (1991) *Biochemistry* 30, 9859-

-
59. Coppolino, M.G., Woodside, M.J., Demarex, N., Grinstein, S., St Arnaud, R., and Dedhar, S. (1997) *Nature* **386**, 843-847
60. Hynes, R.O. (1992) *Curr.Opin.Genet.Dev.* **2**, 621-624
61. Santoro, S.A. (1986) *Cell* **46**, 913-920
62. Languino, L.R., Gehlsen, K.R., Wayner, E., Carter, W.G., Engvall, E., and Ruoslahti, E. (1989) *J.Cell Biol.* **109**, 2455-2462
63. Yokosaki, Y., Monis, H., Chen, J., and Sheppard, D. (1996) *J.Biol.Chem.* **271**, 24144-24150

Figure Legends

Figure 1

Affinity purification and immunoprecipitation of collagen type II-binding integrins from triton X-100 lysate of ^{125}I -labeled human chondrocytes. The lanes show immunoprecipitation of integrins using monoclonal antibodies against the integrin subunits $\beta 1$ (P4C10), $\alpha 1$ (TS2/7), $\alpha 2$ (P1E6) and $\alpha 3$ (P1B5). The proteins eluted by EDTA from the collagen type II-Sepharose are shown in lane E. The proteins were separated by SDS-PAGE (4-12%) under nonreducing conditions and visualised using a phosphoimager.

Figure 2

Affinity purification of the $\alpha 10$ integrin subunit on collagen type II-Sepharose. A triton X-100 lysate of bovine chondrocytes (2.5×10^9 cells) was applied to a fibronectin-Sepharose precolumn followed by a collagen type II-Sepharose column. The lanes show EDTA-eluted proteins from the fibronectin-Sepharose (A), flow-through from the collagen type II-Sepharose column (B) and EDTA-eluted proteins from the collagen type II-Sepharose (C). The eluted proteins were precipitated by methanol/chloroform, separated by SDS-PAGE (4-12%) under reducing conditions and stained with coomassie blue. The 160 kD-protein with affinity for collagen type II is indicated with a arrow.

Figure 3

Schematic map of the sequencing strategy. The overlapping $\alpha 10$ clones hc1 and hc2 were obtained by screening a human articular chondrocyte library with a bovine $\alpha 10$ probe. The Race1 clone was obtained from human chondrocyte cDNA using the RACE technique. Arrows indicate the direction and extent of nucleotide sequencing.

Figure 4

Nucleotide sequence and deduced amino acid sequence of the human $\alpha 10$ integrin subunit. The

amino acid translation is under the first nucleotide of the corresponding codon. The signal peptide cleavage site is marked with a arrow, human homologues to bovine peptide sequences are underlined and the I-domain is boxed. Metal ion binding sites are indicated with a broken underline, potential N-glycosylation sites are indicated by an asterisk and the putative transmembrane domain is double underlined. The normally conserved cytoplasmic sequence is indicated by a dot and dashed broken underline. The sequence data is available from xxxxx under accession number xxxxxx.

Figure 5

Diagrammatic comparison of $\alpha 10$ with the general structure of integrin α -subunits. The conserved repeats 1-7 are indicated with boxes. An I-domain is found in some α -integrin subunits. Other α integrin subunits are posttranslationally cleaved near the transmembrane domain (TM). The integrin subunit $\alpha 10$ contains the 7 conserved repeats and an I domain located between repeat 2 and 3.

Figure 6

Sequence similarity between integrin α -subunits. The similarity tree was constructed by using the GCG software and the program "Pileup". Percent identities were calculated using the Jotun Hein algorithm provided in the Lasergene DNASTAR software. The similarity tree indicates three different subfamilies of α -integrin subunits; one subfamily that contains I-domains ($\alpha 1$, $\alpha 2$, αM , αX , αL and $\alpha 10$), one subfamily that is cleaved ($\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, αv and αIIb) and one subfamily that neither contains I-domains nor is cleaved ($\alpha 4$, $\alpha 9$).

Figure 7

Northern blot analysis of $\alpha 10$ and $\beta 1$ mRNA. Bovine chondrocyte mRNA was hybridized with ^{32}P -labeled cDNA probes corresponding to the integrin subunits $\alpha 10$ or $\beta 1$. The $\alpha 10$ -probe hybridized to an mRNA of 5.4 kb and the $\beta 1$ -probe hybridized to an mRNA of 3.5 kb on the same filter.

Figure 8

Immunoprecipitation of the $\alpha 10$ integrin subunit from human chondrocytes. Triton X-100

lysates of ^{125}I -labelled human chondrocytes were immunoprecipitated with polyclonal antibodies against the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 10$. The immunoprecipitated proteins were separated by SDS-PAGE (4-12%) under reducing (a) and non-reducing conditions (b) and visualised using a phosphoimager.

Figure 9

Immunostaining of human articular cartilage. An antibody raised against the cytoplasmic domain of $\alpha 10$ (see Table II) stained the chondrocytes in tissue sections of human articular cartilage (A). The staining was depleted when the antibody was preincubated with the $\alpha 10$ -peptide (B). A control antibody recognising the $\alpha 9$ integrin subunit did not bind to the chondrocyte (C).

Table I

Amino acid sequences of peptides from bovine $\alpha 10$ -integrin. Peptides were isolated by in-gel digestion with trypsin and sequenced by Edman degradation.

Table II

Comparison of the cytoplasmic tails of I-domain containing integrin α -subunits. The underlined sequence in $\alpha 10$ represents the peptide that was used for antibody-production.

Peptide	Amino acid sequence
1	DNTAQTSAYIQYEPHISI
2	GPGHWDR
3	AAFDGSGQR
4	FAMGALPD
5	FTASLDEWTTAAR
6	VDASFRPQGXLAP

Q1 KIGFFKRPLKKKMEK

Q2 KLGFFKRKYEKMTKNFDEIDEETELSS

Q10 KIGFFAHEKKIPPEEKREKLEQ

Q11 KLGFFKRQYKIDMMSEGGPPGAEPQ

Q12 KVGFFKRQYKEMMEEANGQIAPFNGTQTPSPSEKPSK

Q13 KVGFFKRNLKEKMEAGRGVPNGIPAEISQLASGQEAGDPGCLKPLIEKDSSEGGGKD

Q14 KCGFFKRKYQQINLESIRKAQIKSENILLEEN

Title: Integrin subunit $\alpha 10$

Inventor: Evy Lundgren-Åkelund, Department of Cell and Molecular Biology, Lund University, Sweden

Abstract

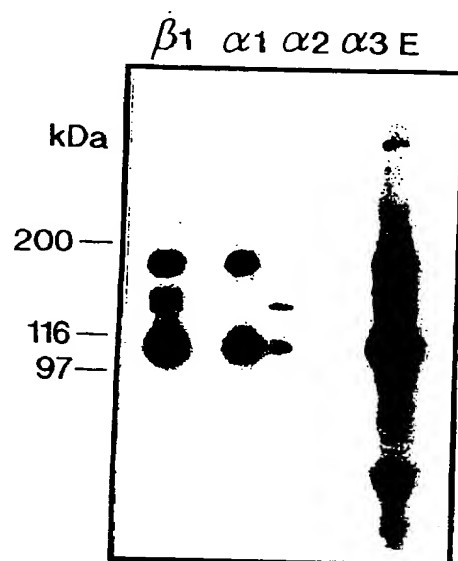
This invention relates to a novel integrin subunit $\alpha 10$ and the use of this integrin as a marker of chondrocytes, smooth muscle cells or endothelial cells. This invention also relates to the use of antibodies recognizing integrin subunit $\alpha 10$ or cDNA encoding for integrin $\alpha 10$ peptides in identifying integrin subunit $\alpha 10$ on chondrocytes, smooth muscle cells and endothelial cells and determining the differentiation state of the cells.

Background of the invention

See enclosed manuscript entiteled "Isolation, cloning and sequence analysis of a the collagen-binding integrin $\alpha 10\beta 1$ expressed on chondrocytes".

Claims

1. Integrin subunit $\alpha 10$ used as a marker of chondrocytes, smooth muscle cells or endothelial cells.
2. The use of integrin $\alpha 10$ antibodies or cDNA encoding for integrin $\alpha 10$ -peptides as markers of the integrin subunit $\alpha 10$ on chondrocytes, smooth muscle cells and endothelial cells.
3. A peptide consisting of the amino acid sequence of the cytoplasmic domain of integrin subunit alpha10, KLGFFAHKKIPEEEKREEKLEQ.
4. Polyclonal antibodies raised against the peptide claimed in 3.
5. The use of integrin $\alpha 10$ -antibodies or cDNA encoding integrin $\alpha 10$ peptides to determine differentiation-state of chondrocytes during development, in pathological conditions such as reumatoid arthritis and osteoarthritis, in tissue regeneration and in therapeutic and physiological repair of cartilage.
6. The use of integrin $\alpha 10$ -antibodies or cDNA encoding integrin $\alpha 10$ peptides to determine differentiation-state of smooth muscle cells in development and in pathological conditions such as atherosclerosis and inflammation.
7. The use of integrin $\alpha 10$ -antibodies or cDNA encoding integrin $\alpha 10$ -peptides to determine differentiation-state of endothelial cells during embryonic development, angiogenesis, or development of cancer.
8. Activation or blockage of the integrin subunit $\alpha 10$ to stimulate extracellular matrix syntesis and repair or to decrease extracellular matrix degradation.



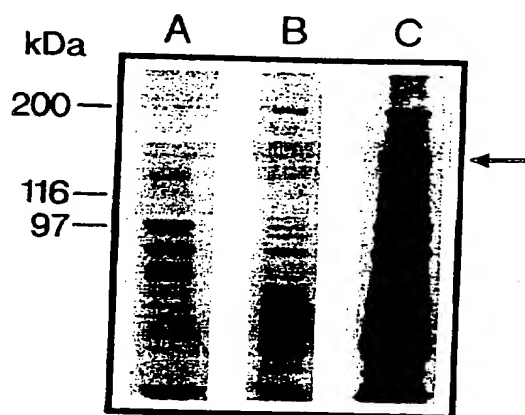
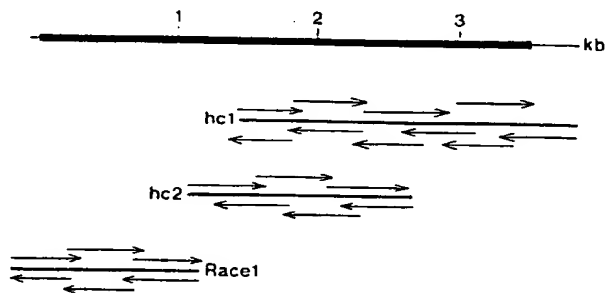


Fig. 3



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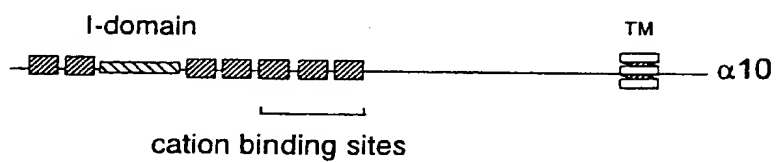
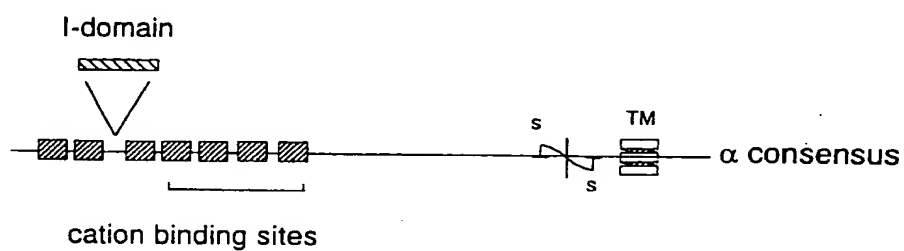
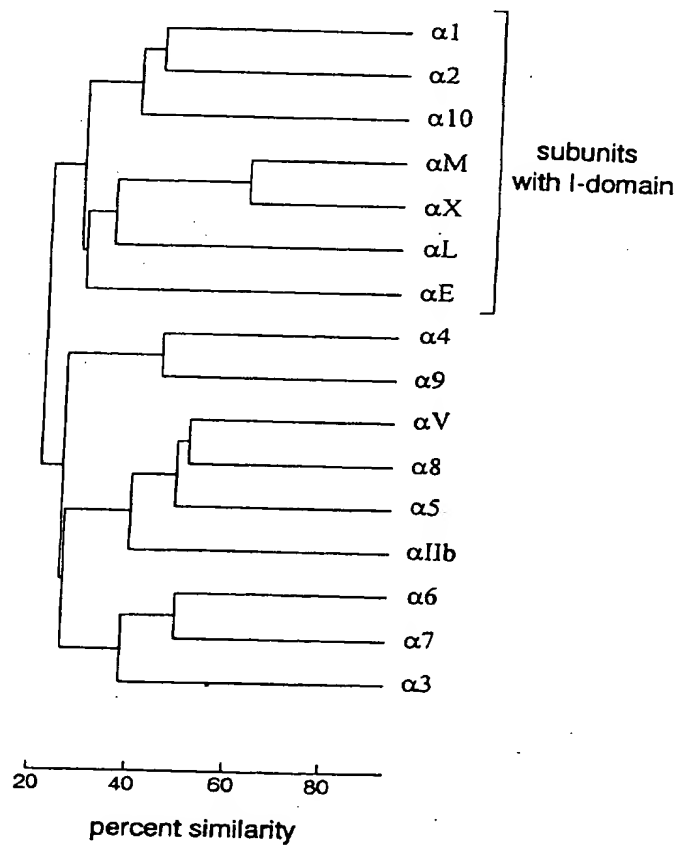


Fig. 6



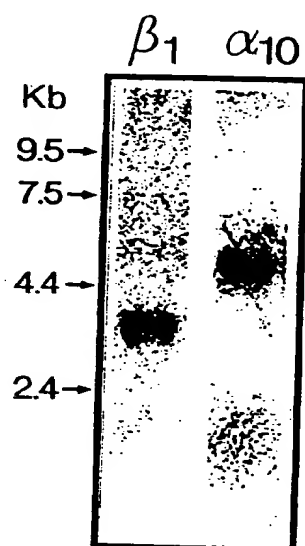


Fig. 8a,b

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